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NATURE OF THE "VITAMIN A-LIKE FACTOR" IN LARD

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ONE FIGURE

It has been shown by Kaunitz and Slanetz ('50a) that the occurrence of vitamin A deficiency in rats can be prevented by feeding them a diet containing a molecularly distilled forerun fraction from lard. Their results showed that this fraction in most instances had biological activity equivalent to abount 5 to 25 units of vitamin A acetate per gram. Assuming that this forerun fraction, which amounted to 7% of the lard, contained nearly all the active material, the potency of the original lards would range from about four-tenths to two units per gram. Chemical and spectroscopic analyses by 4 laboratories, however, failed to establish the presence of conventional vitamin A alcohol or ester. Therefore, it was tentatively concluded that the active material belonged to the somewhat vague group of substances possessing biological A activity but assumed to be chemically different from the known vitamin A. Reports of such substances have appeared within the last two decades (Randoin and Netter, '34; Lemley, Brown,

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Bird and Emmett, '47; Dubouloz, Marville and Chevalier, '48; LeGallic, '48; Mayer and Krehl, '48; Grangaud and Massonet, '49; Lane, '50).

The investigation of the nature of this biologically active material was continued. If the material could be concentrated further by molecular distillation, adsorption fractionation, or crystallization, identification might be established. It was also deemed probable that failure to detect vitamin A by the spectroscopic method could be attributed to the presence of unsaponifiable impurities difficult to separate which have significant absorption in the spectral region, 300 to 325 mμ. The difficulties of separating these absorbing impurities in fish liver oils and fortified margarines have been discussed by Gridgeman et al. ('48); Barua and Morton ('49); Morton and Stubbs ('48); Wilkie ('49); and Boldingh and Drost ('51). The difficulties of separating interfering unsaponifiables from the vitamin factor would be expected to be greater for lard than for fish liver oils, butter or fortified margarines, because the factor is present in much lower concentrations and the ratio of unsaponifiables to units of the factor is also likely to be much greater.

The present paper is a report of an extended investigation into the nature of the active factor in lard. Evidence of the presence of the conventional form of vitamin A was obtained.

EXPERIMENTAL

Materials

Two samples [3] obtained as forerun fractions from molecular distillation of two different batches of freshly rendered lard were available from previous work (Kaunitz and Slanetz, '50b). These were distilled at temperatures of up to 215°C. and 3 μ of pressure. The fractions, amounting to about 7% of the lard, had been kept tightly sealed and under refrigeration and still showed biological vitamin A activity. They will be referred to as original distillates (O.D.-I and O.D.-II).

[3] Distillation Products Industries, Division of Eastman Kodak, and Parke-Davis, Inc., kindly furnished the molecular distillate samples.

The biological activity of O.D.-I was equal to 4.5 units of vitamin A per gram; that of O.D.-II to 25 units per gram.

Bioassay technique for determining vitamin A activity

The bioassays were carried out on albino rats. The mothers of the young eventually used were placed, within three days of the births of their litters, on a highly purified, vitamin A-deficient diet. After weaning at 21 days, the young received a more rigidly vitamin A-deficient diet, consisting of 54% cerelose, 30% alcohol-washed casein, 4% salts (U.S.P. No. 2), 2% celluration, 10% rancid lard and, per kilogram, 5 gm $CaCO_3$, 1 gm inositol, 1 gm choline, 300 mg *p*-aminobenzoic acid and 100 mg nicotinic acid. They were fed separately 4 times weekly two drops of a suspension containing, per milliliter, 20 mg calcium pantothenate, 4 mg thiamine, 8 mg riboflavin, 8 mg pyridoxine, 5 mg folic acid, 50 µg biotin, 10 µg vitamin B_{12} and 50 mg ascorbic acid.[4] The lard was made rancid to destroy the vitamin A which it could have contained; there was no objection to this procedure because it has been proved that mildly rancid lard is not toxic (Stoerk, Kaunitz and Slanetz, '52).

When the animals were 28 days old, groups of 12 matching young were made up. The average weights of the groups at 24 and again at 28 days were identical. To each rat were now given 10 gm of a diet containing, instead of the rancid lard, 1 gm of the experimental fat fraction, or 1 gm of cottonseed oil containing either no vitamin A or a known amount of U.S.P. standard vitamin A acetate. To this were added, in the earlier experiments, 0.1 ml of linoleic acid containing 6.25 mg alpha-tocopherol acetate, 7.5 mg free alpha-tocopherol and 2.5 µg of vitamin D per 150 gm of diet. In the later experiments, 10 times as much of the two forms of alpha-tocopherol was given. The difference in the tocopherol supplement did not change the results.

[4] Dr. Leo Pirk of Hoffmann-La Roche, Inc., very kindly supplied most of the various vitamins for this work.

Forty-eight hours after they had been offered the supplemented diet, the animals had nearly always consumed the 10 gm offered, and they were then fed the rancid lard diet without restriction. Henceforth their weights were recorded every other day, and the maximum weight gains of the individual animals from the day previous to their being fed the supplements until they started losing weight were averaged for each group. When supplements of zero to 50 units of vitamin A acetate (U.S.P.) were fed, there was always a graded weight response. The average weight gain of the unsupplemented group was subtracted from the average weight gain of each of the groups receiving supplements, and the resulting differences were plotted on a log-log scale against the amount of supplement.

In most experiments, there was nearly a straight-line relationship between weight increase and dose, up to a supplement of 25 units. Above 25 units, the slope of the curves decreased. With a single dose of two units of vitamin A acetate, a discernible difference from results with the unsupplemented group could be produced. The probable errors obviously were greater the lower the supplement. They could have amounted to roughly $\pm$ 50% for single doses of two to 5 units and to $\pm$ 25% for supplements of from 5 to 25 units. Actually, however, when a standard curve was established for each assay of materials with unknown activities, the results agreed — perhaps somewhat fortuitously — to within $\pm$ one-half unit per gram. The results were read from the standard curve by interpolation. It must be emphasized that for each series of biological assays a new standard curve had to be prepared.

Spectroscopic determination of vitamin A

The treatment of lard and molecular distillates from lard to obtain the vitamin A factor free of other unsaponifiables which have irrelevant absorption in the spectral region 300 to 325 mμ, was similar to that described by Boldingh and Drost ('51) in their work on margarine. A quantity of sample, estimated from biological assays to contain vitamin A

activity equivalent to about 100 units, was saponified with alcoholic potash, and the unsaponifiables were extracted and chromatographed on alumina and alkaline alumina in accordance with the procedure of these workers. The size of the alumina column (but not that of the alkaline alumina column) was increased in some instances owing to comparatively large amounts of unsaponifiables involved. In other instances eluted fractions, which gave a positive Carr-Price test but which still contained interfering impurities when examined spectrophotometrically, were subjected to a second treatment on fresh alumina and alkaline alumina. Estimations of vitamin A were based only on eluates which gave a positive Carr-Price test and which also showed a maximum in spectral density at 325 mμ. The potency was calculated by multiplying $E^{1\%}_{1\,cm}$ (at 325 mμ) with the established conversion factor of 1,900.

RESULTS AND DISCUSSION

Attempts were made first to determine whether redistillation or crystallization of the original lard distillates would further concentrate the biologically active vitamin A factor.

Treatment of original distillate, O.D.-I

About 525 gm of distillate O.D.-I were subjected to molecular distillation in a cyclic still of the falling film type, about 15% being distilled and removed as 4 fractions, leaving an undistilled portion of 85%. Another portion of O.D.-I, 300 gm, was crystallized from acetone, a precipitate being removed at 0°C. and another at — 45°C., leaving some material as a filtrate fraction. The biological assays for vitamin A activity on these fractions and analyses for unsaponifiable matter are shown in table 1. Distillation was more effective than crystallization in producing a fraction with high biological assay.

These fractions were largely consumed in the biological assays; small amounts remaining were examined spectro-

photometrically without attempts to remove or separate unsaponifiables. Inflections in the spectral density curves at about 325 mμ were obtained, which were only suggestive of the possible presence of vitamin A.

Larger quantities, about 3,000 gm, of O.D.-I were similarly distilled except that about 2.5% was removed as a first fraction (B-1) and about 7.5% as a second fraction (B-2), leaving an undistilled portion (B-3) of about 90%. The biological

TABLE 1

Bioassay for vitamin A on fractions obtained from lard by molecular distillation and by crystallization

SAMPLE NO.		FRACTION	UNSAP.	BIOLOGICAL ASSAY
	Molecular distillation	%	%	*units/gm*
O.D.-I	Fr. 1 – from lard, 210°C. at 3 μ	7	4.2	4.5
A1	Fr. 1 – from O.D.-I, 85– 95°C. at 3 μ	1.5	> 35	0
A2	Fr. 2 – from O.D.-I, 95–125°C. at 3 μ	4.5	> 35	4.5
A3	Fr. 3 – from O.D.-I, 125–165°C. at 3 μ	4.0	33.2	23.0
A4	Fr. 4 – from O.D.-I, 165–200°C. at 3 μ	4.5	7.1	18.0
A5	Fr. 5 – from O.D.-I, undistilled	85	0.4	0
B1	Fr. 1 – from O.D.-I, 85–125°C. at 3 μ	2.5	38.9	2.5
B2	Fr. 2 – from O.D.-I, 125–200°C. at 3 μ	7.5	35.8	11.5
B3	Fr. 3 – from O.D.-I, undistilled	90	0.4	2.6
	Crystallization from acetone			
O.D.-I C1	Precipitate from O.D.-I, 0°C.	24	0.3	2.5
C2	Precipitate from O.D.-I, — 45°C.	61	0.6	2.6
C3	Filtrate from O.D.-I, — 45°C.	15	20.8	11.5

assays for vitamin A activity and values for unsaponifiable material are included in table 1.

Attempts to determine vitamin A in fractions B-1 and B-2 by means of chromatographic fractionation of the unsaponifiables and subsequent spectrophotometric measurements of eluted fractions resulted in only qualitative indications of its presence. Eluates were obtained which gave a weak Carr-Price test and a slight maximum at 325 mμ. The shape of the spectral curves, however, did not closely resemble that of known pure vitamin A because of too great absorption in the region 300 to 320 mμ. It was concluded that in order

to obtain more definite spectral evidence of the presence of typical vitamin A, a sample containing a lower ratio of unsaponifiables to biologically active factor would be required.

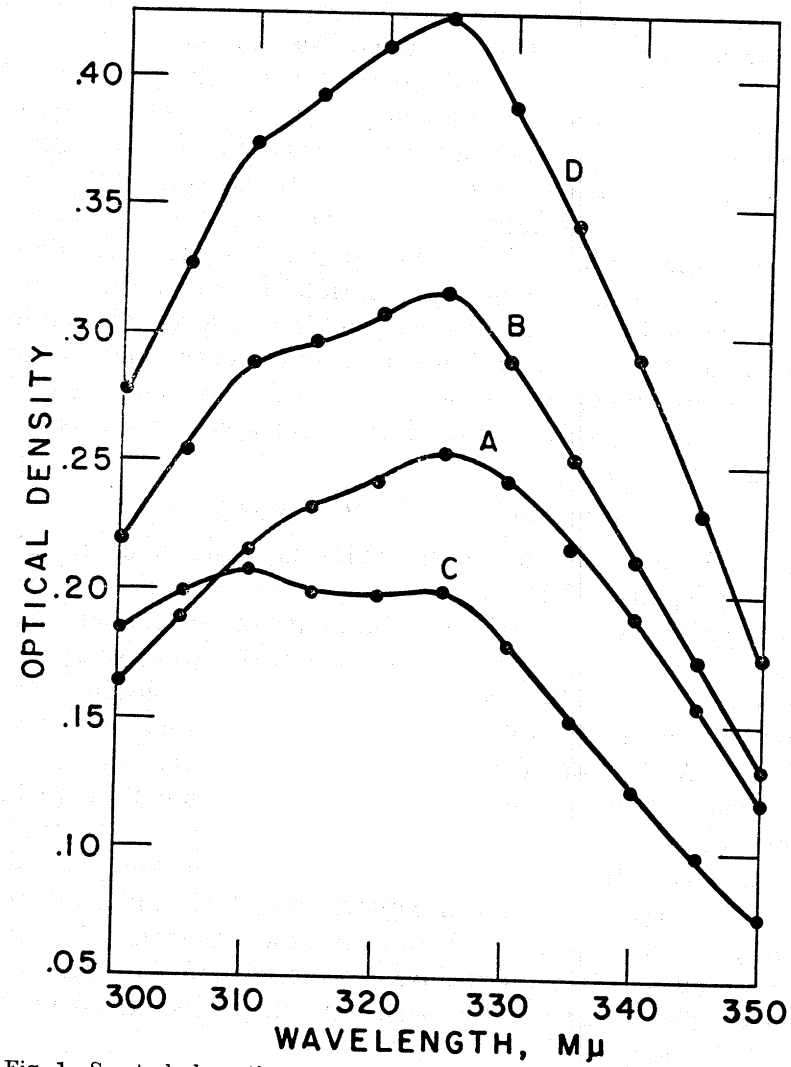

Fig. 1 Spectral absorption curves for standard vitamin A and for eluted fractions obtained by Boldingh and Drost method.

A, U.S.P. vitamin A reference standard; B, distillate from lard (O.D.-II); C, lard; D, lard to which vitamin A was added.

Fraction B-3, although relatively low in the active factor, fortunately was also low in total unsaponifiable matter. A 60-gm portion was saponified and the unsaponifiables extracted and chromatographed according to the method of Boldingh and Drost ('51). Eluted fractions giving positive Carr-Price tests were combined and examined spectrophotometrically. The curves obtained by plotting spectral density against wavelengths were almost identical to curve C (fig. 1). Assuming the absorption at 325 mμ to be due entirely to vitamin A, the calculated potency was two and eight-tenths and one and eight-tenths units per gram on separate determinations. By employing the equation of Morton and Stubbs ('48), which introduces a correction for irrelevant absorption, the values calculated were one and seven-tenths and one and seven-tenths units per gram. These values compare well with the biological assay result of two and six-tenths units per gram (table 1).

Analyses of lard distillate O.D.-II

A sample of lard distillate (O.D.-II) which had biological activity equal to 25 units of vitamin A per gram was available from previous work. It was relatively low in unsaponifiable material, containing only 2.2%. Therefore, this sample appeared to offer the greatest promise of any obtained from lard in this study for establishing the presence of typical vitamin A. The procedure of Boldingh and Drost was applied to 5-gm portions of this sample. Eluates were obtained which gave strong Carr-Price tests and a spectral curve (B) typical for known vitamin A (curve A, fig. 1). The potency calculated for duplicate determinations was 23.8 and 24.3 units per gram of distillate. Employing the equation of Morton and Stubbs ('48), the values were 23.8 and 24.0 units per gram. These values agree well with that determined by biological assay.

Analyses of lard

Biological assays have indicated that different samples of lard usually have vitamin A activity equivalent to about

four-tenths to two units per gram. An attempt was made to apply the Boldingh and Drost method for spectral determination of vitamin A to fresh lard without molecular distillation. For this purpose, 120 gm of lard were saponified, and the unsaponifiables extracted and chromatographed in accordance with the procedure. Eluates were obtained which gave positive Carr-Price tests and a spectral curve (curve C, fig. 1) resembling that of known vitamin A except for slight extraneous absorption in the region 300 to 320 mμ. The potency calculated according to the method was six-tenths units per gram (corrected value, four-tenths units per gram), a value within the range arrived at by biological assay.

It is concluded that typical vitamin A is present in lard and probably accounts for most of its biological vitamin A activity. The possibility of the presence in lard of other substances possessing similar activity but differing chemically from vitamin A is not completely excluded but is considered unlikely.

The finding of vitamin A in lard also renders unlikely previously published assertions that lard has a so-called "sparing" action on added vitamin A when it is isocalorically substituted for other nutrients, such as carbohydrate and protein. The results of this investigation may also raise a reasonable doubt about some of the reports of biological materials which are claimed to have vitamin A activity but which differ chemically from known vitamin A.

SUMMARY

Biological assays on molecular distillates from lard showed that lard contains vitamin A activity equivalent to about four-tenths to two units per gram.

Chromatographic fractionation of unsaponifiables from lard and molecular distillates from lard yielded eluates which gave positive Carr-Price tests and typical vitamin A spectral curves, except in fractions having an extremely high ratio of unsaponifiables to units of vitamin A.

It is concluded that the biological vitamin A activity of lard is largely attributable to the presence of typical vitamin A. The so-called "sparing" action of lard on utilization of added vitamin A in diets is in all probability due to the presence in lard of hitherto unrecognized typical vitamin A.

ACKNOWLEDGMENTS

We are highly indebted to Dr. Waldo C. Ault of the Eastern Regional Research Laboratory and Dr. J. J. Beaver of Columbia University for their interest and advice in connection with this work.

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